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Report

# Replication Fork Stability Is Essential for the Maintenance of Centromere Integrity in the Absence of Heterochromatin

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http://dx.doi.org/10.1016/j.celrep.2013.02.007

## SUMMARY

**The centromere of many eukaryotes contains highly repetitive sequences marked by methylation of histone H3K9 by Clr4$^{KMT1}$. This recruits multiple heterochromatin proteins, including Swi6 and Chp1, to form a rigid centromere and ensure accurate chromosome segregation. In the absence of heterochromatin, cells show an increased rate of recombination in the centromere, as well as chromosome loss. These defects are severely aggravated by loss of replication fork stability. Thus, heterochromatin proteins and replication fork protection mechanisms work in concert to prevent abnormal recombination, preserve centromere integrity, and ensure faithful chromosome segregation.**

## INTRODUCTION

Repetitive sequences generally are unstable and prone to recombination and replication fork stalling (Branzei and Foiani, 2010; Carr et al., 2011; Voineagu et al., 2008, 2009). This can lead to replication-induced gross chromosome rearrangements (GCRs) (e.g., Chen and Kolodner, 1999; Mizuno et al., 2009), which are associated with cancer (Rajagopalan and Lengauer, 2004; Colnaghi et al., 2011). The fission yeast pericentromere consists of repetitive sequences assembled into heterochromatin (Nakaseko et al., 1986, 1987), which is "cold" to recombination (Nakaseko et al., 1987). The loss of heterochromatin structure is with increased meiotic recombination in this region (Zaratiegui et al., 2011; Ellermeier et al., 2010), suggesting that heterochromatin protects the repetitive sequences.

Pericentromere heterochromatin is replicated early in S phase (Kim et al., 2003), and this early timing depends upon the chromodomain protein Swi6 (HP1) (Hayashi et al., 2009; Li et al., 2011b). Swi6 is dislodged from the centromere during mitosis (Kloc et al., 2008; Chen et al., 2008), allowing a window of transcription of small noncoding RNAs. These program the RNAi-associated RITS complex, including the chromodomain protein Chp1, which in turn recruits Clr4 methyltransferase (KMT1) to methylate histone H3K9 and recruit Swi6 again (Noma et al., 2004; Verdel et al., 2004). An alternative mechanism couples DNA polymerase epsilon to the Clr4 complex to ensure that newly synthesized DNA is marked by H3K9me (Li et al., 2011a).

Most heterochromatin mutants have a negative synthetic growth phenotype when combined with mutations that affect regulation of recombination, such as *rad51*Δ or *mus81*Δ (Zaratiegui et al., 2011; Roguev et al., 2008). A simple model suggests that loss of heterochromatin structure alone is sufficient to increase recombination in the repetitive sequences of the centromere. This effect may be exacerbated in the RNAi mutants because of collisions with RNA polymerase II, as has been proposed for *dcr1*Δ mutant by Zaratiegui et al. (2011). However, this mechanism does not explain the increased recombination observed in other heterochromatin mutants, not all of which affect retention of RNA polymerase II.

Integrity of the replication fork is known to be important to preserve stability of repetitive sequences in the euchromatin (Branzei and Foiani, 2010; Carr et al., 2011; Mizuno et al., 2009; Voineagu et al., 2008, 2009). In this report, we use two different reporter systems to show that proteins required for replication fork stability also contribute to integrity of the pericentromeric heterochromatin, an effect distinguishable from the instability associated with the RNAi mutants.

## RESULTS

### Heterochromatin Proteins Inhibit Recombination at the Centromere

We examined rearrangements in a nonessential minichromosome (similar to that in Nakamura et al., 2008; Tinline-Purvis et al., 2009) in strains lacking *swi6* or the RITS subunit *chp1* (Figure 1 and Figure S1). Consistent with previous observations by Doe et al. (1998), we observed a high rate of minichromosome loss in both mutants (Figure 1B). Similar loss rates were observed in *mis4ts* cells with a defective cohesin protein (Figure 1B), consistent with *swi6*Δ and *chp1*Δ reducing centromere cohesion (Bailis et al., 2003; Bernard et al., 2001; Nonaka et al., 2002).

We examined minichromosome rearrangements genetically, using markers on the left (*LEU2*) and right (*hph$^R$*, *ura4$^+$*, and *ade6$^+$*) chromosome arms (Figure 1A). We selected Leu$^+$ Ura$^-$ colonies and screened for the *hph$^R$* or *ade6$^+$*. Around 40% of the Leu$^+$ Ura$^-$ Ade$^-$ colonies in wild-type (WT) cells retain the centromere-proximal *hph$^R$* marker. We performed PFGE on the

WT Leu$^+$ Ura$^-$ Ade$^-$ derivatives under conditions that separate whole chromosomes and identified two classes: those with a minichromosome that is larger than the starting construct (Figures 1C and S1A), and those with a slightly smaller minichromosome (Figure 1D). We found that the larger derivative likely results from break-induced replication (BIR) using chromosome III as a template, and the smaller derivative corresponds to an isochromosome, ChL-Iso, probably formed through recombination in the *imr* repeat, as shown previously by Nakamura et al. (2008). Importantly, the WT derivatives all maintained an intact chromosome III.

In contrast to WT cells, almost none of the Leu$^+$ Ura$^-$ Ade$^-$ strains isolated from *swi6*Δ or *chp1*Δ cells retained *hph*$^R$, suggesting complete loss of the right arm of the minichromosome. In all cases, the derivatives we recovered are significantly smaller than the parental minichromosome, which can be seen using PFGE conditions that separate small fragments (Figure 1D). To elucidate the breakpoint, we examined the structure of the junctions between two domains: the inner repeat and the outer repeat *imr3-otr3* (L1 and R1), and the outer repeat and the euchromatin boundary (L and R), by PCR performed on isochromosomes isolated from the PFGE gel (Figure S1C). Similar to WT (*parental*, *P*), two bands were observed with the *imr3-otr3* junction (L1 and R1) in *swi6*Δ and *chp1*Δ, indicating that both the left and right junctions of the *imr* domain are present, although some of them are heterogeneous in size. However, only the L boundary of the *otr* domain remains. Thus, the isochromosome forms at a breakpoint to the right of the *otr* repeat in *swi6*Δ and *chp1*Δ.

Next, we examined the effect of replication fork integrity on this rearrangement. Mrc1 is required for replication fork processivity and for S phase checkpoint activation (Tanaka and Russell, 2001; Alcasabas et al., 2001; Katou et al., 2003; Xu et al., 2006). Although *mrc1*Δ does not increase minichromosome loss rate compared to WT, it does show a modest increase in gene rearrangement compared to *swi6*Δ or *mis4ts*, measured by recovery of Leu$^+$ Ura$^-$ Ade$^-$ clones. The majority of these clones showed the small size typical of the isochromosome ChL-Iso (Figure 1D). However, the double-mutant *swi6*Δ *mrc1*Δ was distinctly different (Figure 1C). First, the increased chromosome loss phenotype associated with *swi6*Δ was reduced in the double mutant. Second, the number of Leu$^+$ Ura$^-$ Ade$^-$ recombinants recovered was significantly higher than the single-mutant strains, suggesting that instead of chromosome loss, chromosome rearrangements are increased. Only 12% of these were *hph*$^R$. A substantial fraction generated larger minichromosomes. However, unlike WT, in many cases, these were accompanied by loss of the normal chromosome III, consistent with gross chromosome rearrangements or translocations (compare lanes 5 and 6 in *swi6*Δ *mrc1*Δ in Figure 1C). The strain was also extremely slow growing, consistent with increased genome instability.

We could not recover a *chp1*Δ *mrc1*Δ double mutant carrying the minichromosome, suggesting that it is even more unstable than *swi6*Δ strains. We examined plating efficiency of *swi6*Δ or *chp1*Δ single or double mutants in the absence of the minichromosome and found that *swi6*Δ mutants were similar to WT, whereas *swi6*Δ *mrc1*Δ and *chp1*Δ mutants reduced recovery of viable clones to the same extent (about 34%; see Table 1). Recovery of *chp1*Δ *mrc1*Δ was reduced to 16%.

## Loss of Replication Fork Stability Increases Recombination in Heterochromatin Mutants

Consistent with previous observations by Roguev et al. (2008) and Zaratiegui et al. (2011), we observed that *swi6*Δ mutants have a synthetic growth defect when combined with *rad51*Δ or *mus81*Δ (Figure 2A). We constructed double mutants between genes required for heterochromatin assembly (*swi6*Δ, *chp1*Δ, *clr4*Δ, and *dcr1*Δ) and *cds1*Δ (affecting the replication checkpoint kinase), *mrc1*Δ, or a checkpoint-specific allele lacking all phosphorylation sites (which we call *mrc1-SA* here) (Xu et al., 2006). We observed increased sensitivity to both HU and TBZ in all the double mutants (Figure 2A). Interestingly, the checkpoint-specific allele *mrc1-SA* shows very little phenotype compared to the complete deletion of *mrc1*Δ, which also affects replication fork stability and processivity (Xu et al., 2006).

Because the minichromosome assay is unwieldy, we designed a simple substrate to examine recombination in the pericentromeric outer repeats in these double mutants, consisting of the *ura4*$^+$ gene interrupted by the *his3*$^+$ gene (Figure 2B). The two fragments of *ura4*$^+$ contain a 200 bp overlap; a successful recombination event will restore *ura4*$^+$ and delete *his3*$^+$. We inserted this cassette in place of an existing *ura4*$^+$ transgene in the *dh* region of the centromere I (Allshire et al., 1994) (Figure 2B). Importantly, this cassette is silenced in WT cells and only expressed in mutants lacking fully functional heterochromatin (Figure 2C). Thus, its rate of recombination (determined by recovery of Ura$^+$ colonies) can only be examined in the heterochromatin mutants with silencing defects that allow expression of *ura4*$^+$.

Recombination rates at the centromere in *chp1*Δ, *clr4*Δ, and *dcr1*Δ are higher than in *swi6*Δ; this was not a surprise because these three mutations create more severe disruptions in histone methylation and silencing than *swi6*Δ (Schalch et al., 2009). We examined markers of double-strand break response including histone H2A phosphorylation, Rad52 foci, and Chk1 phosphorylation in *chp1*Δ cells (Figures S2A–S2C). We did not see evidence for increased H2A or Chk1 phosphorylation in *chp1*Δ, suggesting that global damage response is not activated (Figure S2C; compare lanes treated with DNA-damaging agents to untreated cells). There was a modest increase in the number of cells with more than one Rad52 focus in *chp1*Δ (Figure S2A).

In *swi6*Δ, *chp1*Δ, or *clr4*Δ mutants combined either with *cds1*Δ or *mrc1*Δ, there was a striking increase in recombination of our test substrate (Figure 2C). Importantly, this increase is occurring in the absence of HU treatment, indicating that replication fork integrity checkpoint mechanisms are required for genome stability during otherwise unperturbed S phase, if heterochromatin is disrupted. Consistent with this, a double-mutant *swi6*Δ *swi1*Δ, which lacks a component of the fork protection complex (Noguchi et al., 2003, 2004) also showed increased recombination. We observed only a slight increase in recombination in *swi6*Δ cells following HU treatment and little if any effect in *chp1*Δ or *clr4*Δ (Figure S2D).

In contrast to the striking increase in recombination observed for *swi6*Δ *mrc1*Δ, *chp1*Δ *mrc1*Δ, or *clr4*Δ *mrc1*Δ, we saw no increase in *dcr1*Δ *mrc1*Δ, although the strain does have a synthetic growth defect. There was only a small increase in recombination in *dcr1*Δ *cds1*Δ. Thus, in our recombination assays, we find

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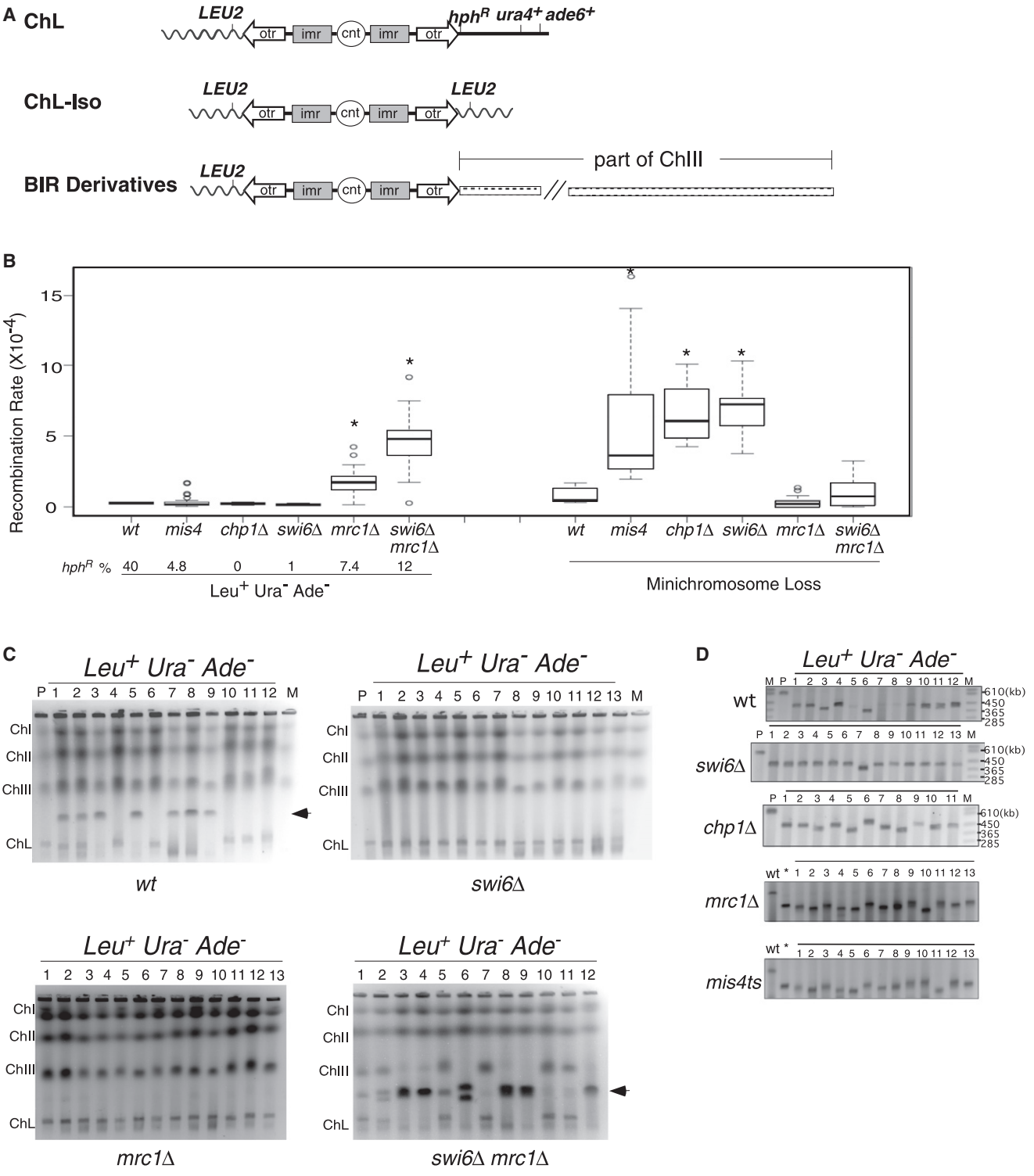


**Figure 1. Heterochromatin Factors and Mrc1 Prevent Rearrangement at *otr* in the Centromere**

(A) The structure of the minichromosome (ChL), modified from Nakamura et al. (2008), isochromosome derivative (ChL-Iso), and BIR derivatives.

(B) Frequency of different gene rearrangement events in WT (FY5187), *swi6*Δ (FY5109), *chp1*Δ (FY5107), *mrc1*Δ (FY6443), *mrs4ts* (FY6447), and *swi6*Δ *mrc1*Δ (FY6506). The bold line in each box represents the median, and the open circles represent outliers in the data set. $hph^R$ is the fraction of *hph*-resistant clones in $Leu^+$ $Ura^-$ $Ade^-$ colonies. Asterisks indicate recombination rates that are significantly higher than WT ($p < 0.05$, Mann-Whitney U test).

(C) PFGE of whole chromosomes from indicated strains of P and $Leu^+$ $Ura^-$ $Ade^-$ colonies shows intermediate-sized chromosomes in *swi6*Δ *mrc1*Δ. Arrowhead indicates BIR derivatives. P, parental strain; M, marker.

*(legend continued on next page)*

that *chp1*Δ behaves more like *swi6*Δ than *dcr1*Δ when combined with *mrc1*Δ.

In WT cells, a brief burst of transcription occurs in the pericentromere during S phase and is required for RNAi-dependent heterochromatin assembly (Volpe et al., 2002). The promoters of the reverse and forward transcripts are distal and proximal to the *dh* replication origin, respectively (Figure S2E), and they show different levels of expression (Kloc et al., 2008; Zaratiegui et al., 2011). We investigated whether destabilizing the replication fork affects transcript levels. In the *clr4*Δ, *dcr1*Δ, or *chp1*Δ mutants, we observed increased levels of pericentromeric transcripts in both directions as expected (Motamedi et al., 2008). In the double mutants, there was modest reduction in forward transcripts in *swi6*Δ, *clr4*Δ, or *chp1*Δ, although this did not reach statistical significance (Figure S2E, upper panel). The trends were broadly similar for the reverse transcripts, and the reduction in *clr4*Δ *mrc1*Δ was significant. In contrast, the double mutants with *dcr1*Δ trended higher than the single *dcr1*Δ, again suggesting different effects of destabilizing the replication fork in the RNAi mutants.

## Double Mutants Have Defects in Chromosome Segregation

Increased rearrangement within the centromere in double mutants is likely to affect chromosome segregation. We examined mitosis in live cells expressing a histone H3-RFP fusion protein, by time-lapse video microscopy (Movies S1, S2, S3, S4, S5, and S6). As cells enter mitosis, their chromosomes condense, forming a granular appearance that we infer corresponds with prophase/metaphase. We found that on average, mitotic *chp1*Δ cells persisted in this state longer than either WT or *swi6*Δ mutants (Figures 3A and 3B). Deletion of *mrc1*Δ modestly increased the time *chp1*Δ cells spent with this appearance but caused a striking increase in *swi6*Δ. Interestingly, in some cases, cells begin chromosome segregation then transiently return to a single nucleus before completing or aborting anaphase (Figure 3A).

We also determined the frequency of lagging chromosomes, apparent anaphase bridges, and uneven segregation in a minimum of 40 independent mitoses for each strain (Figures 3C and 3D). We observed fewer anaphase bridges for *swi6*Δ *mrc1*Δ or *chp1*Δ *mrc1*Δ. On the other hand, we observed higher uneven and lagging chromosome segregation in *swi6*Δ *mrc1*Δ, but not *chp1*Δ *mrc1*Δ. This implies that double-mutant cells linger in metaphase with entangled or nonaligned but condensed chromosomes.

## DISCUSSION

Pericentromeric heterochromatin in fission yeast is transiently disrupted during G1 and S phase, allowing a brief window of transcription (Kloc et al., 2008; Chen et al., 2008). This programs an RNAi-dependent system that recruits the histone methylation machinery and contributes to RNA turnover (Grewal, 2010; Zofall and Grewal, 2006). Cells lacking the RNAi protein Dcr1 are defective in silencing and fail to release RNA polymerase II from the centromere, which leads to collisions between the transcriptional apparatus and the replication fork and increased recombination (Zaratiegui et al., 2011). This is consistent with evidence suggesting that increased formation of transcription-associated R loops throughout the genome causes replication barriers and increased recombination (Bermejo et al., 2011; Domínguez-Sánchez et al., 2011; Gómez-González et al., 2011). Not surprisingly, common fragile sites in the human genome are also collision sites between replication and transcription (Helmrich et al., 2011).

Zaratiegui and colleagues reported that *swi6*Δ cells show fewer fork collisions in their 2D gel assay, which they inferred was due to delayed replication (Zaratiegui et al., 2011). However, loss of other heterochromatin proteins, not just RNAi mutants, leads to destabilization of the repetitive sequences of the centromere, and increased recombination (Nakamura et al., 2008; Ellermeier et al., 2010). We find that there is significant recombination in the outer repeats in both *swi6*Δ and *chp1*Δ. Moreover, although *swi6*Δ mutants replicate the centromere late (Hayashi et al., 2009; Li et al., 2011b), *chp1*Δ mutants replicate early, similar to *clr4*Δ mutants (P.C.L., M.D.G., and S.L.F., unpublished data). Additionally, *dcr1*Δ recombination phenotypes in our study are distinct from those seen for *swi6*Δ or *chp1Δ*. Therefore, we propose that collisions between transcription and replication machineries are not the only contributors to instability in this domain.

Similar to higher eukaryotes, the fission yeast pericentromere regions contain repetitive elements (Nakaseko et al., 1986, 1987). It has been suggested that a recombination-based mechanism is important to maintain the centromere structure and promote proper spindle attachment (McFarlane and Humphrey, 2010). Studies show increased rates of recombination in repetitive sequences when replication forks are destabilized, either by mutation or by drug treatments that stall DNA replication (Branzei and Foiani, 2010; Carr et al., 2011; Voineagu et al., 2008, 2009). Previously, it was shown that loss of the homologous recombination protein Rad51 leads to increased recombination in the *imr* repeats, forming isochromosomes (Nakamura et al., 2008; Tinline-Purvis et al., 2009). Thus, Rad51 may limit inappropriate rearrangements in this domain.

**Table 1. The Plating Efficiency of *ura*$^{-}$-*his*$^{3+}$ *ura4* Cassette Strains on YES**

| Strain | Plating Efficiency (%) |
|---|---|
| FY4166 WT | 75 |
| FY4538 *swi6*Δ | 64 |
| FY4625 *chp1*Δ | 35 |
| FY5691 *swi6*Δ *mrc1*Δ | 35 |
| FY5798 *chp1*Δ *mrc1*Δ | 16 |

(D) Chromosomes were extracted from parental and Leu$^{+}$ Ura$^{-}$ Ade$^{-}$ colonies of indicated strains and separated by PFGE using conditions specific for smaller DNA fragments; these do not resolve the full-length chromosomes (see Experimental Procedures). WT, FY5187. Asterisks indicate WT *Leu*$^{+}$ *Ura*$^{-}$ *Ade*$^{-}$ derivatives.
See also Figure S1.

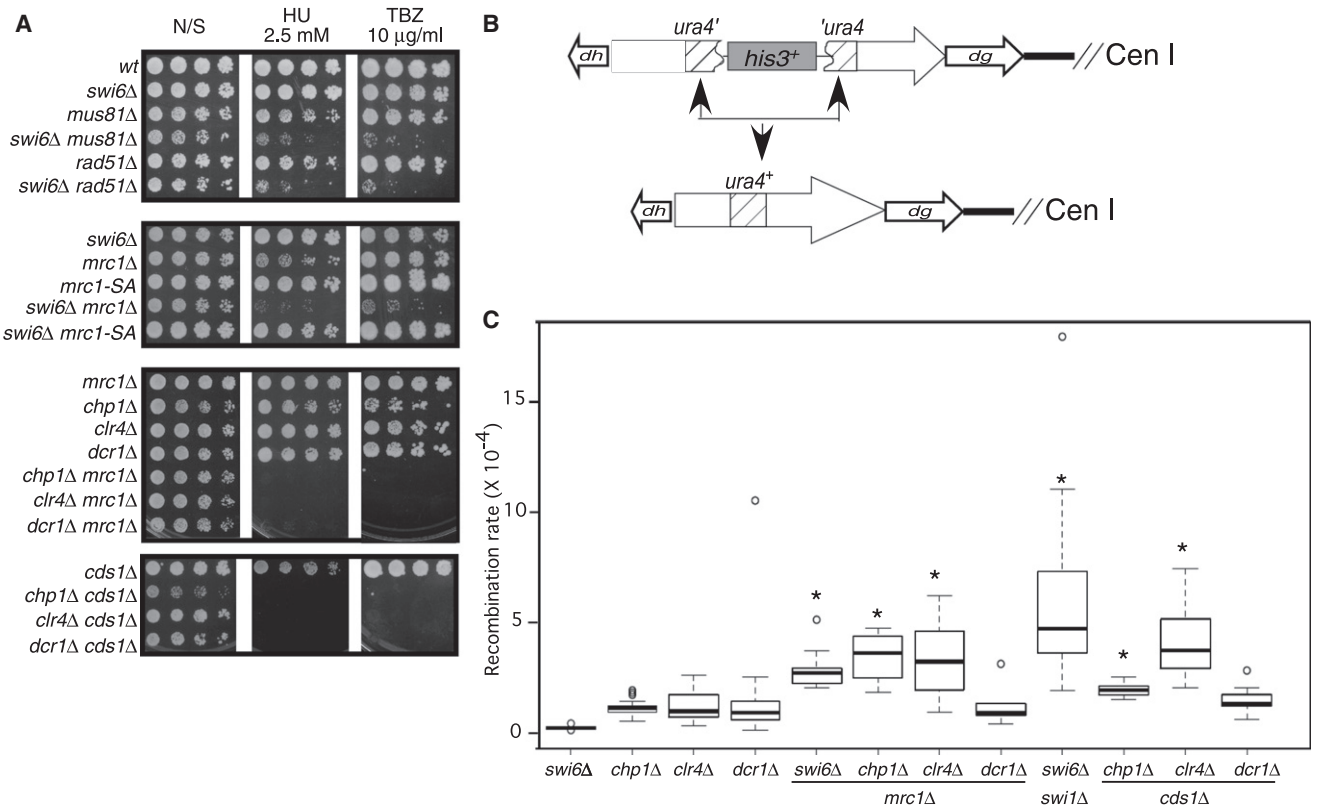


**Figure 2. Double Mutants between Heterochromatin Factors and Fork Protectors Show Higher HU/TBZ Sensitivity and Pericentromeric Recombination Rate**

(A) Cells were grown on nonselective medium (N/S), 10 μg/ml of thiabendazole (TBZ; a spindle poison), or 2.5 mM of hydroxyurea (HU; a replication fork staller) at 32°C with 1:5 serial dilutions.

(B) Recombination of *ura4'-his3*$^{+}$*-'ura4* integrated at the centromere produces Ura$^{+}$ His$^{-}$ colonies in silencing-defective strains.

(C) Recombination rate is calculated from Ura$^{+}$ events by Lea-Coulson method (Hall et al., 2009). For each strain, three independent experiments were performed with five or more colonies in each experiment. The bold line in each box represents the median, and the open circles represent outliers in the data set. Asterisks mark samples with recombination rate significantly higher than the single mutant with $p < 0.05$ (Mann-Whitney U test.).

See also Figure S2.

We used two different in vivo assays to show that loss of Chp1 or Swi6 increases recombination in the *otr* region. Mutations that decrease replication fork stability have a synergistic effect with mutations in *swi6*Δ, *chp1*Δ, or *clr4*Δ, leading to dramatic increases in recombination, chromosome loss, and defects in chromosome segregation. This is not observed when treating the heterochromatin mutants with HU to induce fork stalling, which suggests that as long as normal mechanisms that preserve fork integrity are intact, there is no additive effect.

In fission yeast, the mating locus is another heterochromatin domain that is also H3K9 methylated and Swi6 enriched, although it does not require RNAi-dependent machinery (Petrie et al., 2005). Replication pausing is essential for imprinting and mating-type switching in this region (Arcangioli and de Lahondès, 2000; Kaykov et al., 2004), and this depends upon demethylation of histone H3K9 by Lsd1 (Holmes et al., 2012). This is consistent with our observation that *clr4*Δ mutants are hyperrecombinant. Together, these data indicate that there is an essential interplay between the H3K9 methylation status and the replication fork that fine-tunes genome processing.

The most dramatic phenotype is observed in mutants lacking the fork protection complex proteins Mrc1 and Swi1. These help maintain fork stability and coupling between the leading strand polymerase and the helicase (Noguchi et al., 2003; Matsumoto et al., 2005). A double-mutant *swi6*Δ *mrc1*Δ has a slow growth phenotype, and GCRs involving the minichromosome and chromosome III, which we speculate are likely translocations (detailed mapping of these structures will be presented elsewhere). We were unable to recover the *chp1*Δ *mrc1*Δ strain with the minichromosome, suggesting that it is even more unstable than *swi6*Δ *mrc1*Δ. This is consistent with the increased recombination we see in *chp1*Δ with the *ura4'-his3*$^{+}$*-'ura4* recombination substrate.

However, whereas *chp1*Δ has the highest fraction of abnormal mitoses including anaphase bridges, uneven segregation, lagging chromosomes, and delay with condensed chromosomes, the double-mutant *chp1*Δ *mrc1*Δ actually reduces the number of abnormal segregations. We hypothesize that this is due to increased cell death and to mitotic delay with entangled chromosomes (measured by prolonged condensed chromatin). In contrast, the *swi6*Δ *mrc1*Δ cells show no change in chromosome

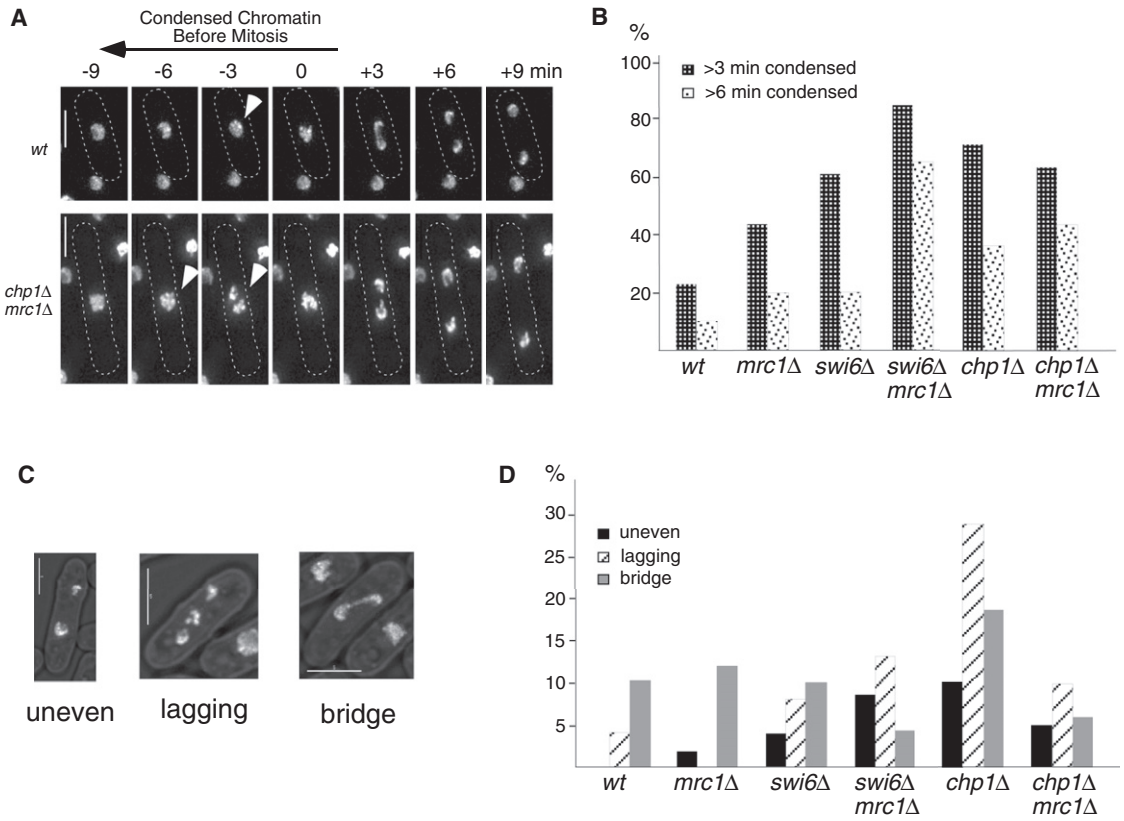


**Figure 3. Mitosis Is Abnormal in *swi6*Δ *mrc1*Δ or *chp1*Δ *mrc1*Δ Mutant Cells**

Histone H3 (*hht1*) tagged with RFP in WT (FY5512), *swi6*Δ (FY6465), *mrc1*Δ (FY5843), *chp1*Δ (FY5315), *swi6*Δ *mrc1*Δ (FY6464), and *chp1*Δ *mrc1*Δ (FY5842) cells. Live-cell images were taken every 3 min at 30°C, and the ten Z section images at each time point were deconvolved, and maximum intensity was projected.

(A) Representative images of WT and *chp1*Δ *mrc1*Δ cells shown with cell outline (dashed lines). White arrowheads indicate condensed chromatin mass before mitosis. Scale bars, 5 μm. *swi6*Δ *mrc1*Δ phenotypes are similar.

(B) Average percentage of cells with either >3 or >6 min duration of condensed chromatin before mitosis.

(C) Representative images of uneven, lagging, and bridge chromosome segregation.

(D) Average percentage of the cells with uneven, lagging, and bridge chromosome segregation. More than 45 cells entering mitosis were analyzed in each experiment, and two independent experiments were examined.

See also Movies S1, S2, S3, S4, S5, and S6.

segregation but a dramatic increase in the time spent with condensed chromosomes. This is consistent with reduced plating efficiency observed in *chp1*Δ *mrc1*Δ compared to *swi6*Δ *mrc1*Δ (Table 1). Both Swi6 and Chp1 are chromodomain proteins that bind H3K9me, although Chp1 does so with higher affinity (Schalch et al., 2009).

Our results are consistent with the chromosome aberrations observed in solid tumors, especially in BRCA1-deficient breast and/or ovarian cancers, which affect heterochromatin assembly and DNA repair, and loss of genome stability (Zhu et al., 2011). Indeed, recent work suggests that a combination of mitotic defects including lagging chromosomes contributes to formation of DNA damage and chromothripsis in micronuclei that is associated with cancer (Guerrero et al., 2010; Crasta et al., 2012). Our work suggests that heterochromatin factors and replication stability proteins function synergistically to maintain the stability of the centromere and potentially other repetitive or fragile domains to prevent such rearrangements.

## EXPERIMENTAL PROCEDURES

All the strains used in this study are listed in Table S1.

### Construction of *ura4′-his3⁺-′ura4* Cassette at the Centromere Region

The 5′ UTR and 1–500 bp ORF of ura4⁺ were amplified from plasmid pRIP4 using primers #1113 and #1114 by Expand Polymerase (Roche) and cloned into KpnI-SalI-digested plasmid pAF1 to create pRCP15. The 300–795 bp ORF and 3′ UTR of *ura4*⁺ were amplified using primers #1124 and #1125, digested with Pst1 and SacI, and cloned into pRCP15 to generate pRCP16. The cassette was released by SacI and KpnI digestion, purified by gel extraction, and transformed into FY4294 to create FY4538. Derivatives were constructed by standard genetics.

### Rate of *ura4′-his3⁺-′ura4* Recombination and GCR-Minichromosome Loss

Strains were grown in selective PMG (*Pombe* Glutamate Medium; Sabatinos and Forsburg, 2010) to midexponential phase. Cells were diluted to rich medium (YES) at 100 cells/μl and cultured for eight to nine generations to

allow spontaneous recombination. A total of 2.5 × $10^5$ cells were plated on PMG−Ura on 15 cm Petri dishes and grown for 4–5 days at 30°C. A total of 1,000 cells were plated on YES medium to calculate the plating efficiency. The recombination rate was calculated using Lea-Coulson method (http://www.keshavsingh.org/protocols/FALCOR.html), and statistics were computed by Mann-Whitney U tests (http://vassarstats.net/).

### PFGE and PCR Analysis

PFGE was performed using CHEF-DR III Variable Angle System (Bio-Rad) as described in Pankratz and Forsburg (2005). To separate whole chromosomes, we used 120°, 1,200–1,800 s switch time, 72 hr, 14°C, 2 V/cm, 1% of Megabase Agarose in 1× TAE. To examine the minichromosomes, we used 120°, 60–120 s switch time, 24 hr, 14°C, 6 V/cm, 1% of Megabase Agarose in 0.5× TBE. The minichromosomes were extracted from the gel as in Thuring et al. (1975), and the recovered DNA was suspended in 100 mM Tris-HCL (pH 7.5). All PCRs were performed using Accuprime System (Invitrogen; 12339-016) using the primers described in Nakamura et al. (2008). The PCR products were separated by 1× TAE, 2% agarose.

For additional details, please see the Extended Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, two figures, one table, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.007.

## LICENSING INFORMATION



## ACKNOWLEDGMENTS

We thank J. Cooper for the red histone, T. Nakagawa for the ChL strain, T. Kelly for the *mrc1-SA* strain, R. Allshire for strains, and members of the lab for assistance and helpful comments on the manuscript. This work was supported by grants to S.L.F. from NSF MCB 0743448 and NIH R01 GM059321. R.C.P. was supported by the American Cancer Society (grant no. PF-09-113-01-GMC) and NIH training grant 1T90DE021982-01.

Received: August 1, 2012
Revised: January 6, 2013
Accepted: February 5, 2013
Published: March 7, 2013

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